THE EFFECT OF TOLUOL AND CS_2 UPON THE MICRO-FLORA AND FAUNA OF THE SOIL.[1]

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The work herein presented was undertaken to determine whether the theory advanced by Russell and Hutchinson (1) was adequate to explain the phenomena subsequent to "partial sterilization" when applied to local soils. As is well-known, this theory rests upon the destruction of protozoa that inhibit the maximum development of soil bacteria and hence prevent the rendering of soil nitrogen available. Certain results obtained have been of such a nature that the writer feels justified in bringing them before the public. This is done with the hope that investigations along the same lines by others may either controvert or substantiate the facts herein presented. These investigations are by no means complete, and hence this paper is more of the nature of a preliminary report.

The recent appearance of Fred's (2) article, in which an excellent review of most of the literature bearing upon the subject was given, renders a complete historical résumé unnecessary, hence only a summary of the more important contributions will be given. Readers are referred to the article just mentioned for a more complete review.

The evidence brought forth thus far as to the effects of "partial sterilization" upon the microörganisms of the soil has, with one or two exceptions, been very fragmentary. Most investigators have concerned themselves with the effects of such treatment upon crop production. However, the writer has been able to glean from the literature the following expressions regarding such treatment upon the more important soil phenomena.

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[1]Abstracted from a thesis presented to the Faculty of Washington University, in candidacy for the degree of Master of Arts, June, 1911.

(a) A temporary decrease in total number of bacteria present, with a subsequent large increase: Krüger and Heinze (3), Massen and Behn (4), Pfeiffer (5), Hiltner and Störmer (6), Russell and Hutchinson (1), Hutchinson (7), and Fred (2).

(b) An increase in oxidation: Fischer (8), Hesselink von Suchtelen (9), and Darbshire and Russell (10).

(c) An increase in ammonification: Störmer (11), Scherp (12), Laidlow and Price (13), Lipman (14), and Russell and Hutchinson (1).

(d) A detrimental effect upon nitrification: Warington (15), Scherp (12), Laidlow and Price (13), Perraud (16), Pfeiffer (5), Wagner (17), and Russell and Hutchinson (1).

(e) A beneficial effect upon nitrification: Brailles (19), Wollney (20), Pagnoul (21), Störmer (11), Hiltner and Störmer (6), Coleman (22), Lipman (14), Koch (23), and Fred (2).

(f) A detrimental effect upon nitrogen fixation: Störmer (11), Koch (23), Massen and Behn (4), and Koch and associates (24).

(g) A beneficial effect upon nitrogen fixation: Kramskii (25), Heinze (26), Lipman (14), and Fred (2).

(h) A detrimental effect upon denitrification: Wagner (17), Hiltner and Störmer (6), Störmer (11), Lipman (14), and Fred (2).

(i) In regard to the effect upon nodule organisms: Wollney (20), and Nobbe and Richter, believed such beneficial, while Perrotti (28), Koch (23), and Fruwirth (29) held to the opposite view.

In addition to the above, Hiltner and Störmer, and Russell and Hutchinson have studied with some care the flora, as a whole, prior and subsequent to treatment; and they found that certain types were detrimentally and others indirectly beneficially affected. Russell and Hutchinson have also studied, more or less, the effect of such treatment upon the micro-fauna and, as a result, claim that all types of animal

life, with perhaps one exception, are totally destroyed. Fred, in addition to his study with soils, studied certain types of soil organisms in liquid cultures and found that the addition of certain strengths of various antiseptics stimulated development. This strength varied with different organisms and the stimulative effect diminished gradually from the maximum in both directions.

EXPERIMENTAL.

The work outlined in this paper has to do primarily with the effect of CS_2, toluol, and, to a less extent, chloroform, upon the number of bacteria and the number of types of animal life before and after the application, in varying quantities, of such substances to soils.

EFFECT UPON THE NUMBER OF BACTERIA.

In studying the effect of such chemicals upon the number of bacteria in soils, the following methods were employed: the sample of soil to be studied was carefully freed from worms, etc., and sieved to either remove or break up all large particles, then thoroughly mixed. Sufficient water was added with that already present to bring the content up to one-third or one-half saturation, usually one-half. The desired quantity was then weighed into a sterile dish, care being taken to prevent contamination. The desired quantity of chemical was then added and mixed in; the dish was covered with a glass to prevent evaporation and set aside at room temperature for the desired length of time. In some experiments the treated soil was removed to sterile bottles, in which case the bottles were tightly stoppered with cork or even sealed. No differences were to be noted following these slightly different methods. The per cent of chemical added was in every case based upon dry weight of soil—dried at 110° C. for two hours.

Analyses of all soils were made as follows: the complete sample of soil was thoroughly mixed with a sterile spatula and several small portions taken from different parts of

the dish. This composite sample was again thoroughly mixed and several smaller quantities removed, and from this lot, after a second thorough mixing, the final weighing was made. Weighing was done on analytical balances, a sterile piece of paper being placed upon the pan; and in every instance, one gram of soil was taken. The gram of soil was at once removed to a 125 cc. rubber-stoppered Erlenmeyer flask containing 99 cc. of sterile water. This was shaken vigorously three times for one-third minute each, and while still in motion 1 cc. removed, by means of a sterile pipette, to another water blank. This process was continued until the sample was so dilute that a Petri dish containing 1 cc. could be counted. Duplicate platings were made of the final dilution, using approximately 8 cc. of agar. At first, standard nutrient agar + 1 was used, but this was soon replaced with the synthetic agar proposed by Lipman. Cultures were incubated at room temperature until maximum counting development was attained. With nutrient agar the period was usually three days; with Lipman's synthetic agar one week. Counting was done with a x10 hand lens; and if duplicates varied widely, they were discarded. Only averages are given.

In all, twenty samples of soil have been subjected to one or all of the three chemicals mentioned for periods varying from one day to six weeks. The samples represent soils from rich garden, orchard, forest, lawn, greenhouse, and pasture.

Table I contains a summary of all samples tested, while Table II contains a very conservative illustration of actual results. Table III contains the results of seven samples analyzed after being subjected to different quantities of CS_2 and toluol for three days. Table X contains complete results of the twenty samples tested.

TABLE I.

SHOWING THE NUMBER OF SAMPLES TESTED WITH VARYING STRENGTHS OF TOLUOL, CS_2, AND CHLOROFORM AND THE NUMBERS GIVING INCREASE, DECREASE, AND NO CHANGE IN BACTERIAL CONTENT.

	Toluol				CS_2				$CHCl_3$			
%	S.	S.I.	S.D.	?	S.	S.I.	SD.	?	S.	S.I.	S.D	?
.01	5	4	1	0	5	2	0	3	4	1	0	3
.1	5	3	0	2	5	4	1	0	4	2	0	2
.2	9	8	0	1	7	7	0	0	....	....	....	....
.3	1	1	0	0	1	1	0	0	....	....	....	....
.4	1	0	1	0	2	2	0	0	....	....	....	....
.5	1	1	0	0	1	0	1	0	....	....	....	....
1.	13	5	8	0	13	5	7	1	7	1	6	0
4.	6	2	4	0	6	3	3	0	....	....	....	....
5.	5	0	5	0	3	0	3	0	3	1	2	0
10.	7	0	7	0	7	0	7	0	7	0	6	1
20.	2	0	2	0	2	0	2	0	2	0	2	0

S. = samples tested. S. I. = samples showing an increase.
S. D. = samples showing decrease. ? = samples showing no change.

TABLE II.

SHOWING THE EFFECT OF TOLUOL, CS_2, AND CHLOROFORM UPON THE NUMBER OF BACTERIA IN A SOIL WHEN EVAPORATION WAS PREVENTED.

	Toluol				CS_2				$CHCl_3$			
%	1d.	3d.	7d.	42d.	1d.	3d.	7d.	42d.	1d.	3d.	7d.	42d.
Chk.	3.8	4.7	8.8	3.4	3.8	4.7	8.8	3.4	3.8	4.7	8.8	3.4
.01	5.5	8.1	14.3	6.6	7.5	8.5	7.3	4.7	3.8	6.2	5.8	2.9
.1	3.7	4.5	28.0	14.4	4.3	6.4	4.7	1.5	3.5	5.5	4.9	4.4
1.	2.4	1.8	3.0	2.4	1.9	3.6	5.6	5.5	2.4	2.7	3.3	2.0
10.	2.2	2.6	2.1	1.3	2.0	1.9	2.5	1.5	2.4	2.6	2.2	1.8
20.	1.6	1.4	2.9	1.1	2.0	2.3	2.2	2.5	1.9	2.5	2.4	1.9

Figures=million bacteria per gram soil.
1d., 3d., etc.=days after treatment.

TABLE III.

SHOWING THE NUMBER OF TYPES OF PROTOZOA AND NUMBER OF BACTERIA AFTER SUBJECTING SAMPLES OF SOIL TO TOLUOL AND CS_2 FOR THREE DAYS.

	Types Protozoa Present							Bacteria — Million Per Gram Soil						
Treatment	Chk.	T. .2%	T. 1.%	T. 4.%	C. .2%	C. 1.%	C. 4.%	Chk.	T. .2%	T. 1.%	T. 4.%	C. .2%	C. 1.%	C. 4.%
Soil S. 1....	7	3	0	0	6	5	0	2.0	2.0	2.0	1.6	2.8	1.4	1.0
" " 2....	7	7	....	3	8	....	5	13.2	23.2	.....	31.3	16.3	.....	7.6
" " 3....	7	7	....	4	10	....	6	14.8	22.8	.....	2.8	15.6	.....	23.2
" " 4....	9	9	1	1	9	7	1	5.4	7.3	30.8	4.5	6.5	8.4	19.6
" " 5....	6	5	0	0	4	2	2	11.4	14.4	15.2	40.0	16.3	17.0	8.6
" " 6....	6	7	2	0	7	5	5	5.0	17.4	7.1	4.8	12.4	62.5	27.0
" " 7....	8	*7	7	....	*8	5	....	13.9	*42.0	8.6	.....	21.1	*34.5	....
Total Types	12	11	7	4	12	10	6	.....	.....	.....	.....	.....	.....	....

T. = toluol; C. = CS_2; S. = sample.

*By error .3% was added.

The principal facts brought out in these four tables may be summarized as follows:—

(1) The chemicals studied when applied to soil in small quantities exert a stimulative effect upon the multiplication of aerobic bacteria, capable of developing upon nutrient agar or Lipman's synthetic agar.

(2) This stimulative effect may make itself markedly evident as early as twenty-four hours after treatment, and may remain evident as long as forty-two days—the longest trial period—even when evaporation is prevented.

(3) This stimulative effect is markedly evident with .01%—the weakest trial quantity—and may be evident with 4%, there being apparently a wide variation with different soils.

(4) The greatest stimulative effect lies apparently near .2% for the majority of soils tested, and beyond 1% we usually get a decrease.

(5) The decrease, when evident at 1.%, is nearly as marked with that amount as with 20%.

The above results were at such variance with those obtained by Russell and Hutchinson, the only persons, so far as the writer is aware, that have made laboratory investiga-

tions, even when their work was duplicated as nearly as possible, that a cause was looked for in the period at which analyses were made. While in their work it is not stated whether analyses were made before or after evaporation of the chemical, presumably it was after. To determine this point an experiment was made which gave the following results:—

Check not evaporated .....10.2 million.
.2% Tol. not evaporated.....41.6 "
.2% " after evaporation....00.56 "

As to whether this is the explanation, the writer is unable to say.

Russell (30) further states that "many if not all the active forms of bacteria are also killed" by the treatment. To determine whether this were true, even when a decrease was noted, analyses of a sample were made after treatment, but before evaporation, and again after evaporation. In the evaporation of this sample, the water content fell from 10% to 3%, and in Table IV is given the percentage decrease due to this loss of water.

TABLE IV.

SHOWING THE PERCENTAGE REDUCTION IN NUMBER OF BACTERIA AND ACTUAL REDUCTION IN TYPES OF PROTOZOA DUE TO EVAPORATION OF CHEMICAL AND MOISTURE.

Treatment	% Decrease of Bacteria	Protozoa Present	
		Before	After
Check	48	6	7
CS_2 0.2%	68	6	5
CS_2 1.0%	60	4	3
CS_2 4.0%	72	0	0
Tol. 0.2%	61	3	3
Tol. 1.0%	..	0	0
Tol. 4.0%	73	0	0

CS_2 1.%, CS_2 4.%, and Tol. 4% showed a decided decrease in number of bacteria during exposure to chemical.

If all vegetative cells were killed by the chemicals in question, a reduction of the per cent of water from 10 to 3 in the short space of 48 hours should not give us a decrease in number of organisms. From these results and conclusion (5) above, it seems evident, that even when there is a reduction it only affects certain species and not the vegetative cells of all species. This is in accord with the results of Hiltner and Störmer, and in part with those of Russell and Hutchinson in that they found quite a difference in the types of organisms subsequent to treatment.

Greenhouse Experiments.

In Table V is given an excellent and typical example of the effect of the substances studied on the number of bacteria in soils under greenhouse conditions.

TABLE V.

SHOWING THE BACTERIAL CONTENT OF A SERIES OF GREENHOUSE PLATS DURING THE GROWTH PERIOD OF A CROP, WITH THE RELATIVE YIELD OF THE SAME.

Date	2-21	2-25	3-4	3-11	3-18	4-3	4-18	5-10	Total Yield	Grain Yield
Chk.	.81	.63	5.50	4.60	1.60	2.70	4.10	2.00	100	100
Sterilized	00	.....	130.00	174.00	106.00	102.00	50.00	83.00	155	213
Pt. St.	.12	1.90	39.00	91.00	42.00	106.00	141.00	116.00	209	259
T. 22.5cc.	.81	.74	2.40	2.50	.74	3.40	4.00	4.00	102	96
T. 62. cc.	.81	1.50	3.10	1.00	2.10	4.00	2.20	2.00	123	158
T. 310. cc.	.81	.75	5.40	5.40	2.10	6.70	7.10	2.70	141	180
Chk.	.81	.63	5.50	1.60	2.40	2.20	3.30	1.20	100	100
Sterilized	00	.....	130.00	140.00	74.00	163.00	159.00	35.00	79	36
Pt. St.	.12	1.90	39.00	68.00	22.00	86.00	74.00	38.00	136	68
CS_2 22.5cc.	.81	1.30	3.00	.93	.85	4.40	4.20	2.00	113	119
CS_2 62. cc.	.81	.93	.70	1.70	.82	3.90	2.90	1.80	144	150
CS_2 310. cc.	.81	.73	3.90	1.50	.68	3.50	5.80	6.70	73	80
$CHCl_3$ 62. cc.	.81	.....	1.00	.53	1.00	2.60	2.90	.....	.....	.....

Figures on left of double line = bacteria in million; those on right relative yield. Plats above line seeded to oats, those below to buckwheat. T. = toluol. Pt. St. = partially sterilized.

This experiment consisted of twenty-five plats 1 x 3 feet in size and six inches deep. They were filled with a pasture soil (as poor as could be had) and treated in fours,

with the exception that only one was treated with chloroform. The soil in four plats was sterilized in autoclav (tests showing sterilization to be complete). Four were filled with soil partially sterilized, *i. e.*, heated to a temperature of 90°—100° C. in autoclav without pressure. The chemicals were added in three holes at equal distances apart and in three strengths: 22.5 cc. approximating 7 cc. per square foot or a weak practical application, however, one with which other experimenters have obtained marked increases in yield; 62 cc. or approximately .2% of dry weight of soil; and 310 cc. or approximately 1.% dry weight of soil. After allowing the chemicals to act for four days, the plats were thoroughly stirred; twelve were seeded to oats and thirteen to buckwheat. Duplicate plats were separated as far as possible.

Three samples were taken from the whole depth of duplicate plats by means of sterile tin cylinders. These samples were then thoroughly mixed and analyses made as previously given. Table V gives the bacterial count at intervals, from beginning until just before harvest, also the relative yield of duplicates, both in total dry weight and seed. There were some slight variations in the water content at times, but this was not sufficient, in any of the plats treated, to cause a difference in bacterial content.

From this experiment, typical of others and selected because of the more marked increase in yield and wider variation in treatment, but one conclusion can be drawn: *That with this soil no increase in bacterial content was evident with the wide variation in applications, but, at the same time, a marked increase in yield was obtained.*

With heat, the results were different. There was an enormous increase in number of bacteria, not, however, necessarily concomitant with an increase in yield. In the case of this soil, there were evidently toxic substances produced in the process of heating, for most of the young oat plants died as a result, and those that did not, made no growth for several weeks. With buckwheat, the plants were unable to overcome this detrimental effect in time to show

beneficial results. Similar toxic effects have been reported by Lyon and Bizzell (31), Schulze (32), and Pickering (33).

TABLE VI.

SHOWING THE PRESENCE OF DIFFERENT TYPES OF PROTOZOA AND NUMBER OF BACTERIA FOLLOWING TREATMENT OF GREENHOUSE PLATS.

Treated	Types Protozoa Present 12-15-'11												Bacteria—Million Per Gram Soil	
12–7–'11	a	b	c	d	e	f	g	h	i	j	k	Total	12–15	12–29
Check.....	+	+	+	+	+	+	+	+	—	+	—	9	9.0	7.0
CS_2 37cc.....	+	—	+	+	+	+	+	+	—	+	—	8	13.6	11.6
Tol. 37cc.	+	+	+	+	+	+	+	+	+	—	—	9	10.8	11.2
CS_2 350cc. ..	+	—	+	+	+	+	+	+	+	—	—	8	17.4	15.7
Tol. 350cc...	+	+	+	+	+	+	+	+	+	—	—	9	13.7	19.5

+ = present; — = absent.

In Table VI is given another series of greenhouse plats in which soil was taken from an uncultivated orchard and was by no means a poor soil. The weaker application represents approximately 10 cc. per square foot, while the heavier represents approximately 100 cc. per square foot, or .3%. In both cases the antiseptic was mixed in the soil when applied. Here we have an unmistakable initial stimulative effect on number of bacteria. These plats are still under observation.

From these results the conclusion seems justifiable: *That under the experimental conditions given above the chemicals studied in quantities equivalent to 100 cc. per square foot, or 1.%, do not exert a diminishing effect upon the number of bacteria, but may or may not exert a stimulative effect.*

Field Experiments.

About May 15, 1911, four series of plats (S. I, S. II, S. III, and S. IV), comprising a total of 140 plats, were laid out in the Missouri Botanical Garden. Each plat measured 5 x 10 feet and was surrounded by a trench one foot wide

and five inches deep. The plats in S. I, S. II, and S. III, were treated in duplicates of seven, as follows: check, toluol 1 cc. per square foot, toluol 15 cc. per square foot, CS_2 1 cc. per square foot, and CS_2 15 cc. per square foot. S. IV was subdivided into three series and treated as follows: *(a)* in duplicate, check, toluol 1, 5, 10, 15, and 30 cc. per square foot; *(b)* singly, check, CS_2 1, 5, 10, 15, and 30 cc. per square foot; *(c)* in triplicate, check, toluol 1 and 15, and CS_2 1 and 15 cc. per square foot. The chemicals were added in one hole per square foot punched to the depth of five inches.

S. I was seeded to oats; S. II to buckwheat; S. III to corn; and S. IV to millet.

Unfortunately, no examinations of soil for bacteria were made until August 19, hence the initial effects are unknown. Table VII contains the results of such examinations as were made. Samples for these analyses were taken two or three in number from each duplicate plat and treated, as previously given.

TABLE VII.

SHOWING THE NUMBER OF TYPES OF PROTOZOA AND NUMBER OF BACTERIA IN FIELD PLATS THREE TO FOUR MONTHS AFTER TREATMENT.

Types of Protozoa Found

Soil Treated 5–15–'11	Tol. cc. Per Sq. Ft.						CS_2 cc. Per Sq. Ft.					
Sampled	Chk.	1	5	10	15	30	Chk.	1	5	10	15	30
S. II 8– 9–'11...	9	8	...	...	9	...	.....	9	...	...	9	...
S. III 7–28–'11...	6	6	...	...	8	...	.....	6	...	...	7	...
S. IV 8–31–'11...	7	7	8	8	9	8	8	7	7	7	7	7
S. IV 9–11–'11...	9	8	8	8	9	8	9	9	7	8	9	7
Total No. Types..	9	8	8	8	9	8	9	9	7	8	9	7

TABLE VII—Continued.

Number Bacteria Per Gram Soil

Soil Treated 5–15–'11	Tol. cc. Per Sq. Ft.						CS_2 cc. Per Sq. Ft.					
Sampled	Chk.	1	5	10	15	30	Chk.	1	5	10	15	30
S. II 8– 9–'11..	8.1	8.9	...	...	9.2	....	8.1	9.6	...	....	9.6	....
S. III 7–28–'11..	10.6	10.8	...	...	12.7	....	10.6	8.8	...	....	8.4	....
S. IV 8–31–'11..	6.8	9.2	8.5	8.2	9.0	10.5	7.9	10.8	7.8	15.1	10.9	11.3
S. IV 9–11–'11..	6.8	7.7	7.5	7.7	5.0	7.7	5.8	4.2	9.9	9.2	6.6	2.7

S. IV samples include both *(a)* and *(b)*.

In addition to the above, a series of small plats of the same soil were treated to determine the first effects of the treatment. The quantities added with record of examinations made are given in Table VIII.

TABLE VIII.

SHOWING THE NUMBER OF TYPES OF PROTOZOA AND NUMBER OF BACTERIA IN FIELD PLATS FOLLOWING TREATMENT.

Treated 7–26–'11	Types Protozoa			Bacteria Per Gram	
Treatment	8-31 '11	9–7 '11	Total	8–31 '11	9–7 '11
Check	6	9	9	13.8	16.8
T. 10cc. per sq. ft.	6	7	7	32.4	14.4
T. 50cc. " "	7	6	7	32.4	50.6
Check	4	8	8	14.9	13.3
CS_2 10cc. per sq. ft.	6	6	7	89.0	18.1
CS_2 50cc. " "	7	6	7	14.2	16.2

Figures for bacteria equal million.

A third series was treated on the College Farm, University of Missouri, and a record of treatment together with results of examinations made thus far is given in Table IX.

TABLE IX.

SHOWING NUMBER OF TYPES OF PROTOZOA AND NUMBER OF BACTERIA IN FIELD PLATS FOLLOWING TREATMENT.

Plats Treated 11-8-'11	Tol. cc. Per Sq. Ft.					CS_2 cc. Per Sq. Ft.				
Sampled	Chk.	1	10	25	50	Chk.	1	10	25	50
				Types of Protozoa Present						
11-15-'11	9	7	9	9	9	9	6	7	8	7
12- 1-'11	11	11	10	9	7	12	10	11	6	8
12-14-'11	11	10	9	9	8	9	10	7	7	8
				Bacteria Per Gram Soil						
11-15-'11	14.0	14.2	18.9	16.8	.....	14.0	16.0	15.6	10.0	13.3
12- 1-'11	15.6	14.2	18.0	18.6	15.3	19.2	14.3	14.2	.....	39.9
12-14-'11	5.2	4.9	6.4	7.3	23.5	6.0	5.4	5.7	6.7	6.8

Figures for bacteria equal million.

From these results the conclusion seems justifiable: *That while the chemicals used may exert a slight stimulative effect upon the multiplication of bacteria subsequent to treatment, all effects of this action were, in the soil studied, lost within three months time.*

TABLE X.

SHOWING THE NUMBER OF BACTERIA IN TWENTY SAMPLES OF SOIL AFTER BEING SUBJECTED TO VARYING QUANTITIES OF TOLUOL, CS_2, AND CHLOROFORM FOR VARYING PERIODS OF TIME.

Sample	1	2	3	4	5	6	7	8	9	10	10	11	12	13	13	14	14	14	14	15	15	16	17	18	19	20
Days Treated	3	3	3	3	3	3	3	3	3	1	7	3	3	3	14	1	3	7	42	1	7	3	3	3	3	3
Chk	6.6	5.4	14.0	13.2	14.8	11.4	5.0	2.0	66.0	79.8	53.9	84.0	27.5	1.4	1.2	3.8	4.7	8.8	3.4	5.1	6.3	9.8	7.4	6.9	6.7	3.4
Tol. .01%										88 2	55.7			1.2	1.0	5.5	8.1	14.3	6.6						12.9	
Tol. .1 %										92.4	79.0			1.3	1.0	3.7	4.5	28.0	14.4						18.7	
Tol .2 %	10.3	7.3		23.2	22.8	14.4	17.4	2.0	103.0																	9.8
Tol .3 %			42.0																							
Tol. .4 %													14.0													
Tol. .5 %																									13.7	
Tol. 1. %		30.8	8.9			15.2	7.1	2.4		6.7	47.0			0.9	1.1	2.4	1.8	3.0	1.8	2.4	2.2	4.8	3.4		12.6	
Tol. 4. %		4.5		31.3	2.8	40.0	4.8	1.6																		
Tol. 5. %																				2.8	3.2	2.9	3.5		3.4	
Tol. 10. %										5.9	5.0			1.0	0.6	2.2	2.6	2.1	1.3	2.6	2.5	1.8	3.3			
Tol. 20. %																1.6	1.4	2.9	1.1							
CS_2 .01%										80.0	49.1			1.3	1.2	7.5	8.5	7.3	4.7					6.7		
CS_2 .1 %										119.3	61.3			1.8	1.7	4.3	6.8	4.7	1.5					5.3		
CS_2 .2 %		6.5		16.3	15.6	16.3	12.4	2.8																		5.8
CS_2 .3 %			21.1																							
CS_2 .4 %												104.0	54.0													
CS_2 .5 %																								5.1		
CS_2 1. %		8.4	34.5			17.0	62.5	1.4		37.1	57.1			1.6	5.0	1.9	3.6	5.6	5.5	4.9	5.4	5.0	5.8	5.2		
CS_2 4. %		19.6		7.6	23.2	8.9	27.0	1.0																		
CS_2 5. %																				2.3	1.8	7.3	3.8			
CS_2 10. %										3.5	2.8			0.8	0.6	2.0	1.9	2.5	1.5	2.3	2.2	6.7	3.4			
CS_2 20. %																2.0	2.3	2.2	2.5							
$CHCl_3$ .01%										77.7	50.6			1.4	3.7	3.8	6.2	5.8	2.9							
$CHCl_3$ .1 %										92.4	48.0			1.4	1.2	3.5	5.5	4.9	4.4							
$CHCl_3$ 1. %										6.7	44.0			1.0	.9	2.4	2.7	3.3	2.0	2.5	6.0	11.3	3.3			
$CHCl_3$ 5. %																				2.1	2.1	12.3	2.5			
$CHCl_3$ 10. %										2.8	39.0			1.0	.8	2.4	2.6	2.2	1.8	2.6	1.8	9.5	2.2			
$CHCl_3$ 20. %																1.9	2.5	2.4	1.9							

Figures = million per gram moist soil.

EFFECT ON PROTOZOA.

The casual observation of the abundance of certain types of protozoa in cultures of *Azotobacter* from soils previously "partially sterilized," led the writer to undertake a series of experiments to determine whether subjecting local soils to the treatment described by Russell and Hutchinson actually freed them of protozoa. This series of experiments has brought forth some very startling and interesting results, compared with the results of the above mentioned writers.

The same soils as mentioned under the bacteriological part of this paper were also investigated as to protozoa. The methods used were as follows, and while they were by no means satisfactory, they are the best the writer has been able to devise, and do reveal certain facts that seem worthy of mention:—

Samples of treated soil were mixed with twice their weight of sterile water or culture medium in an Erlenmeyer flask, thoroughly shaken, and set aside at room temperature to incubate. At first 20 grams were used, later 50 grams. In the first work, only water cultures were made, but it was later found that if a culture medium, composed of 1% hay decoction (1% hay infusion heated one-half hour in Arnold sterilizer, filtered and sterilized) and 5% white of egg, was used, certain types of protozoa, notably *Amoeba, Colpoda,* and certain flagellates that escape observation in water cultures, would develop in large numbers. No doubt if other media were employed other types would be observed. Now every sample is cultured both in water and in the medium given above.

After incubating the water cultures for twenty-four hours, 5 or 10 cc. of the supernatant liquid were removed (without stirring) with a sterile pipette to a sterile centrifuge tube. The centrifuge was then run until experience had shown practically all protozoa to be thrown down; 1 to 5 minutes usually sufficed. This can be determined by examining the upper portions of the liquid. A sterile pipette was inserted to bottom of tube and sediment removed and

examined microscopically. If examined earlier than twenty-four hours, the particles of soil held in suspension interfered with the examination. Examinations were continued at intervals for at least a week. It was found useless to examine the cultures in media under one week's incubation as the same types appeared there as in water cultures. After the first week such cultures were examined at intervals for two weeks. Often the cultures in media were so overrun with one or two types that development of other types must have been inhibited. A summary of results obtained thus far is given in Table III.

Where a "type" is spoken of, it simply means that those organisms were so different morphologically that they could be easily distinguished under the microscope from the other "types" observed. In some instances the term "type," as here used, undoubtedly includes two or more species. Some of the more common types appearing have been recognized as *Amoeba, Colpoda, Vorticella, Strombidium,* and *Dileptus.* In some instances, a rotifer and nematode are included, but since these organisms do not encyst they should be more sensitive to such substances, hence their inclusion should not invalidate the results obtained. The letters *a-b,* etc., in Table VI refer to "types" and show their presence following different treatment.

From this it appears: (1) *That in the seven soils studied CS_2 and toluol in a strength of .2% do not exert a diminishing effect upon the number of types of protozoa present;* (2) *That in strengths of 1.% and 4.% the chemicals studied do exert a slight diminishing effect upon the number of types present, but that several types (7 with toluol 1.%, 4 with toluol 4%, 10 with CS_2 1.%, and 6 with CS_2 4.%) are able to withstand these strengths.*

Greenhouse Experiments.

The same samples taken from greenhouse plats as referred to in Table VI, taken eight days after treating, were also examined for protozoa and the results of this examination are recorded in the same table.

From this we can draw but one conclusion, *i. e., That with the soil studied under the conditions of this experiment, quantities of CS_2 and toluol equivalent to 100 cc. per square foot, or .3%, even when stirred in, did not, within the first eight days, exert any influence upon the number of types of protozoa observed in the soil.*

Field Experiments.

The same samples of soil that were collected for bacteriological examinations from the three series of field plats given in Tables VII, VIII, and IX were also examined for protozoa and the results of such examinations are recorded in the same tables.

From these results the conclusion that *CS_2 and toluol, in quantities of 50 cc. per square foot when applied to the soils under study, had no diminishing effect upon the number of types of protozoa present* seems permissible. Such decreases as may appear in certain instances are well within the limit of error with the methods used.

GENERAL DISCUSSION.

In endeavoring to correlate the results just given with those previously reported along the same lines, certain difficulties are encountered. First, in regard to the initial effect of an application of the chemicals studied. Hiltner and Störmer, and Russell and Hutchinson have reported a very marked decrease, the former in field, and the latter in laboratory experiments, while the writer, applying the same quantities, has always noted either no effect or an increase in the number of bacteria present. The same investigators noted a subsequent enormous increase, while the writer has been unable to detect an increase that could not be attributed to the stimulative effect of the substance, unless such were added in amounts far exceeding those reported as used. If, however, in laboratory experiments quantities sufficiently large be used, such a decrease, followed by the subsequent increase, will be had. As pointed out

earlier, this difference, at least so far as initial effect is concerned, may be due to the time at which analyses were made, the process of evaporating the substance having been shown to decrease the number of bacteria from 50 to 75 per cent. The apparent correlation between the quantity of chemical required to give a marked decrease in protozoa and the quantity required to give a subsequent increase in bacteria would appear to support the theory of Russell and Hutchinson. This supports with equal strength, though, the theory of Greig-Smith (34) for an increase in quantity, within limits, would effect an equally increasing solution of toxic substances.

So far as the writer is aware, no one has reported a stimulative effect of small quantities of such chemicals (acting in soil) upon number of bacteria, but in the light of Fred's recent work such is not at all surprising. He has shown, beyond a doubt, the stimulative effect upon certain soil organisms when in a liquid medium. And while the quantities acting as a stimulant in soils are much greater than in liquids, in the light of the work of Lipman, and Stevens and Withers (35) this is not surprising, as they have shown that the activities of a complex soil culture in soils may be decidedly different from the activities of the same culture in liquid.

This stimulative effect may or may not be evident under greenhouse and field experimental conditions. If evident, the increase occasioned by it is soon lost. If the theory of Russell and Hutchinson be correct, the above contradictory results are easily explained, for in no case, under greenhouse and field experiments, have the chemicals studied exerted an appreciable effect on the protozoa, and in laboratory experiments, not with the quantities used (.2%) by these investigators (see Tables VI, VII, VIII, and IX).

Measuring the effect produced upon the total number of bacteria by the crop yield, greenhouse experiments have given negative results. In Table VI it is shown that, following applications far exceeding any field experiments thus far reported, no increase in bacterial content was

observed, while the increase in yield was as great as 80%. On the same soil, destruction of protozoa by heat followed by an enormous increase in bacterial content was not, in the case of buckwheat, followed by an increased yield. This may be attributed to the production of toxic substances, but, with oats, the toxic effect was even more marked at the beginning than with buckwheat, and still the increase in yield in one instance amounted to 159% on heated soil.

The writer's results show, as pointed out by Russell and Hutchinson, and Goodsey (36), that numerous types of protozoa are to be found in all soils examined. They do not show, though, that treating such soils with CS_2 and toluol, as has been done in experimental work or with the quantities used by the above mentioned writers (.2%), render such soils free from protozoa. To the contrary, they show no appreciable diminution in number of types present. This is true of laboratory, greenhouse, and field experiments. There is no doubt that sufficiently large quantities will destroy the protozoa, but such quantities are far beyond those of experimental practice. No explanation of why Russell and Hutchinson failed to observe protozoa following treatment can be offered, unless it be that their methods of examination were at fault.

The writer's results thus far regarding the effect of such treatment upon bacterial phenomena other than total number have been too meagre to report. However, the evidence of other writers seems to leave no doubt but that those activities which are of practical moment, namely: ammonification, nitrification, nitrogen fixation, and denitrification, are beneficially effected. (See Lipman, Russell and Hutchinson, and Fred.)

While protozoa have been shown to be universally present in soils, universally beneficial results do not follow the application of such chemicals. In the large series of plats (140), mentioned earlier in this paper, treated with amounts of CS_2 and toluol varying from 1 to 30 cc. per square foot, no beneficial results were observed with any test crops.

Similar negative results have been reported by many investigators.

Thus far, no method has been devised whereby the total number of protozoa can be determined; but, from observation of hundreds of samples, the total number of the different types seems to be very small in normal soils. The method used affords ample time for multiplication, and still the examination of 10 cc. of the supernatant liquid of a twenty-four-hour-old water culture (50 parts soil to 100 parts water) often reveals less than twenty-five organisms. When we take into consideration the low water content so often present in our soils, with the necessarily thin films of water and the small number of comparatively large organisms that must cover this soil, the assumption seems unfounded that such organisms prevent the multiplication of bacteria. In fact, computing the diameter of the theoretical water pore of a medium soil by Slichter's (37) formula, we find that for protozoa to pass through the soil they must pass through holes the diameter of which is many times less than the diameter of their bodies. While the writer does not wish to imply that it is in practice impossible for these flexible organisms to move about in the soil, he does wish to call attention to the fact that with the relatively few active organisms present, the physical conditions of the soil must render their coming in contact with a majority of soil bacteria highly improbable. Furthermore, the recent work of Goodsey speaks strongly against the existence in soils of protozoa in the active condition, at least under normal conditions. If this be true, their effect upon the bacterial content of the soil is nil. Under the conditions cited by Russell and Gadding (38), it seems possible that protozoa might become sufficiently numerous to produce an appreciable effect, but this is only an isolated abnormal condition.

After the body of this article had been written and conclusions drawn, the writer noted with a great deal of pleasure the following conclusions drawn by Emmerich, Leinigen and Loew (40), after investigating the same problem:

"So richtig auch Halli Thoris in vielen Fällen sein wird, so dürfte es doch nicht angänglich sein, dieselbe zu verallgemeinern."

GENERAL CONCLUSIONS.

From the evidence given above, the following conclusions appear justifiable:—

1. That small quantities of CS_2, toluol, and chloroform, such as have been used practically and experimentally, when applied to the soils studied, exert a stimulative rather than a diminishing effect upon the total number of bacteria present.

2. That an application of such quantities of CS_2 and toluol does not have an appreciable effect upon the number of types of protozoa present in such soils as have been studied.

3. That a very marked increase in yield may be noted following such an application when no evident change occurs in total number of bacteria present.

4. That, in the light of the recent work of Koch, Egoroo, Goodsey, Fred, and others, with results presented in this paper, the theory advanced by Russell and Hutchinson to account for the increased yield following the application of such chemicals, appears not tenable for general application.

The writer wishes to express his appreciation to Dr. William Trelease, Director of the Missouri Botanical Garden, who placed unlimited means at his disposal for carrying on this piece of work: to Dr. George T. Moore, at whose suggestion the work was undertaken and whose untiring directions have rendered the results obtained possible: and to Dr. W. C. Curtis, who placed, without reserve, his laboratory and equipment at the writer's disposal for carrying on the protozoalogical end of the work.

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